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The legacy of mixed planting and precipitation reduction treatments on soil microbial activity, biomass and community composition in a young tree plantation

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ABSTRACT

Drought events are expected to increase as a consequence of climate change, with the potential to influence both plant and soil microbial communities. Mixed planting may be an option to mitigate drought stress to plants, however, the extent to which mixed planting mitigates the indirect effect of drought (reduced plant-derived carbon input) on soil microorganisms remains unknown. Using soils from a young experimental plantation in Central Europe, we investigated whether mixed planting (oak monoculture, and oak admixed with 1–3 other tree species) under simulated drought (50% precipitation reduction for 2 years) influenced soil microbial activity, biomass and community composition. To focus on legacy effects - i.e. indirect effects mediated by plant composition and a history of drought, rather than direct effects of reduced water availability - soils were measured at a standardised moisture content (28 ± 1% water holding capacity). Rates of bacterial growth and respiration were lower in soils with a legacy of drought. In contrast, fungal growth was not affected by a history of drought, suggesting that fungi were less adversely affected by reduced plant-input during drought, compared to bacteria. The effect of drought on the fungal-to-bacterial growth ratio was influenced by mixed planting, leading to a disproportionate decrease in bacterial growth in drought-exposed soils under oak monoculture than when oak was admixed with two or three different tree species. The presence of a particular tree species (with specific functional traits) in the admixture, rather than increased tree richness *per se*, may explain this response. Microbial biomass parameters, reflecting both the direct and indirect effects of past drought conditions, were consistently lower in drought-exposed soils than controls. While bacteria were more sensitive to the indirect effect of drought than fungi, the biomass concentrations suggested that the direct effect of reduced moisture affected both groups similarly. Overall, our findings demonstrate that drought can have lasting effects on microbial communities, with consequences for microbial function. Results also suggest that admixing oak with other tree species may alleviate the drought-legacy effect on bacteria and increase tolerance to future drought.

1. Introduction

Drought events are expected to increase as a consequence of climate change (IPCC, 2013). More frequent and intense periods of drought will influence soil microbial communities directly due to reduced water availability (Borken et al., 2006; Sheik et al., 2011; Manzoni et al., 2012; Canarini et al., 2017), as well as indirectly via drought effects on plants (Fuchslueger et al., 2014). During drought, plant productivity is typically reduced, resulting in lowered carbon (C) input to soil (Ciais et al., 2005; Peñuelas et al., 2007; Ruehr et al., 2009; Wu et al., 2011; Hasibeder et al., 2015) from both aboveground litterfall and belowground roots and root-exudation (Jones et al., 2009). According to the principle of niche complementarity (Tilman, 1999; Hooper et al., 2005), mixed planting may be an option to mitigate drought stress to plants, as the response to environmental change is expected to differ in a mixture of several species with different functional traits and strategies for resource utilisation than the same species in monoculture (Forrester and Bauhus, 2016). Several studies suggest that facilitative processes and niche complementarity, driven by functional dissimilarity, in mixed species stands often lead to higher rates of biomass production, tree growth and C sequestration compared to monoculture, especially under drought stress (Lebourgeois et al., 2013; Pretzsch

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et al., 2013; Mina et al., 2017). Differences in plant community composition also affect the composition of soil microbial communities (Thoms et al., 2010; Scheibe et al., 2015; Gunina et al., 2017), demonstrating a clear link between above- and belowground communities. Yet, while mixed planting may mitigate drought stress to plants, the extent to which species mixing can also mitigate the indirect effects of drought (i.e. reduced plant input) on soil microorganisms remains unknown. As effects of mixed planting during drought vary among studies, there is currently insufficient information available to define the expected effect size of mixed planting under drought on microbial communities and microbially-mediated processes.

In addition to the direct effects of reduced water availability during drought (Manzoni et al., 2012; Meisner et al., 2017), a history of drought can also influence microbial mineralisation rates (Evans and Wallenstein, 2012; Allison et al., 2013; Hawkes et al., 2017; Martiny et al., 2017). Historical droughts may affect present-day microbial processes, due to persistent abiotic changes caused by drought, or through drought-related changes in microbial community composition. In one pan-European study, however, no legacy effect of long-term moderate drought (30% precipitation reduction during summer growing season) on microbial activity and composition was observed (Rousk et al., 2013).

Fungi and bacteria have been shown to respond differently to drought (Bapiri et al., 2010; Yuste et al., 2011; de Vries et al., 2012). Fungi may be more tolerant to reduced water availability during drought (Harris, 1981; Manzoni et al., 2012; Guhr et al., 2015). Moreover, bacteria are often considered to be more dependent on labile plant-derived C input from roots (Singh et al., 2006; Bird et al., 2011; Andresen et al., 2014). Consequently, a reduction in plant-derived C input during drought is expected to affect bacterial communities more than fungal communities (Fuchslueger et al., 2014).

To investigate whether mixed planting mitigates against the effects of drought on soil microorganisms, we used soils from an experimental plantation with an oak-admixture gradient in Belgium under simulated drought (two years of 50% precipitation reduction). The oak-admixture gradient provided the opportunity to compare soils from under oak monoculture with soils where oak was growing together with one to three other tree species (hereafter referred to as differences in “tree species admixture; TSA”). Rather than assessing the direct influence of reduced soil moisture on microbial processes, here we evaluated the influence of a legacy of drought on soil microbial processes and community composition (Rousk et al., 2013). Hence, microbial activity and community composition in control and historically drought-exposed soils were measured at the same optimal soil moisture content (28 ± 1% water holding capacity). We expected that (1) process rates would be lower in soils with a legacy of drought, as plant input to soil is often reduced during drought (lower availability of labile C). Moreover, we predicted that (2) there would be a greater effect of drought on bacterial compared to fungal communities, as bacteria are often considered to be more dependent on labile C input from plants. According to the theory of niche complementarity, mixed planting should maintain plant productivity, and thus sustain the plant-derived C input to soil, even under drought conditions. We therefore expected that (3) the drought-legacy effect on microbial growth and respiration would be more pronounced in soils under monoculture compared to mixed species stands. Microbial biomass was also measured, integrating the recent history of environmental conditions and thus reflecting both the direct (reduction in soil moisture) and indirect (reduced C input) effects of past drought. Thus, we predicted that (4) microbial biomass would be lower in soils with a history of drought, due to the long-term effect of lower net microbial growth under drought conditions.

2. Materials and methods

2.1. Soils and field treatment

The study site was located at the Zedelgem site of FORBIO plantations in Belgium belonging to the worldwide Tree Diversity Network (http://www.treedivnet.ugent.be/; Verheyen et al., 2016). Zedelgem is one of the three FORBIO experimental plantations (Verheyen et al., 2013). The site is close to the North Sea (51°9′ N 3°7′ E), with a mean annual precipitation of 855 mm and an average air temperature of 10.5 °C (1981–2010). The site was previously agricultural land and was planted with five locally adapted tree species in the winter of 2009–2010. Soils have been classified as relatively dry sandy soil (Podzol) to moderately wet loamy sand (Gleysol) (Verheyen et al., 2013) according to the IUSS Working Group World Reference Base for soils (2006).

The FORBIO plantations follow a synthetic community approach using a fixed species pool of five tree species (Verheyen et al., 2013). Five site-adapted but functionally dissimilar tree species were planted. The species pool includes *Quercus robur* L. (hereafter oak), *Fagus sylvatica* L. (hereafter beech), *Betula pendula* Roth (hereafter birch)., *Tilia cordata* Mill. (hereafter lime), and *Pinus sylvestris* L. (hereafter pine). Monocultures and admixtures of two to four tree species were planted on the environmentally homogeneous site: all five monocultures, all five possible four-species combinations and a random selection of five two- and three-species combinations. Trees were planted in monoculture patches of 3 × 3 trees with a distance of 1.5 m between each tree. Vegetation in the understorey was inventoried after establishment of the plantation in 2011, revealing that most species were typical of a moist, nutrient rich grassland environment (Verheyen et al., 2013). In the first 3 years following planting, the understorey was mown once each year but has since been unmanaged. In the plots used for this study, dense tall grasses form the understorey, with no other woody shrubs present (M. M. Rahman, personal observation). Consequently, vegetation in each plot is determined by the planted tree composition, and the grass-dominated understorey associated with these trees.

A precipitation reduction (hereafter “drought”) experiment was started in April 2015, to assess the performance of oak and beech saplings under drought conditions. Three drought and three control subplots of 3 m × 3 m for each tree species admixture level were established around oak and beech trees in the south-east side of the FORBIO plantation (Rahman et al., under review). This experimental set-up created monoculture-admixing gradients, with oak and beech trees surrounded only by oak and beech, respectively, as well as oak and beech trees surrounded by one, two or three other species (Supplementary Fig. S1). Here we use soils from the oak-admixture gradient, and henceforth we refer to 1–4 levels of tree species admixture (TSA) (Table 1). As this design is not fully-factorial, i.e. all combinations of different tree species in mixtures are not considered, TSA effects cannot be directly attributed to differences in species richness, and findings must be interpreted in light of tree species composition within admixtures, and the associated understorey.

The drought experimental treatment has been described in detail elsewhere (Rahman et al., under review). Briefly, precipitation was reduced by installing rain exclusion shelters that consisted of PVC gutters (c. 12 cm wide) placed at intervals of c. 25 cm. To promote drainage, a slope was constructed by placing the gutter at a height of 0.95 m from the ground at the upper side and 0.75 m from the ground at the lower side. A 6 m long gutter was placed at the lower side to channel the intercepted water away from the plot. The gutters covered approximately 50% of the subplot area. The amount of precipitation intercepted by the shelters was assessed over 44 days (mid-August to end September 2016), by placing rainfall collectors under and outside the rain shelter in monoculture, two-species admixture and three-species admixture plots. From this assessment, the total incoming precipitation excluded by the shelters ranged between 45 and 55%.

In addition to the rain exclusion shelters installed in drought plots, three subplots of the same size but with reverse gutters (no precipitation interception) were also installed. Since there was no difference in soil temperature among control, drought and reverse subplots (data not shown), it is reasonable to conclude that rain shelters did not have a

Table 1
Tree species composition in experimental subplots.

No. tree species in admixture	Plot no.	Species composition
1	15	oak
1	15	oak
1	15	oak
2	14	oak, beech
2	17	oak, birch
2	17	oak, birch
3	3	oak, beech, pine
3	7	oak, birch, lime
3	18	oak, lime, pine
4	6	oak, beech, lime, pine
4	12	oak, beech, birch, lime
4	16	oak, beech, birch, pine

Plot no. corresponds to plot number from Zedelgem site of the FORBIO plantation (see Verheyen et al., 2013).

substantial shading effect. During visits to the field site, any litterfall that had been intercepted by the shelter was removed and replaced in the litter layer. However, few leaves were caught in the gutters, and as the plots were in the middle of the forest and surrounded by dense canopy it is unlikely that leaves were blown off the shelter, hence the potential for litter loss as a result of the shelter was considered negligible (M. M. Rahman, personal observations).

2.2. In-situ soil temperature and moisture

Soil temperature (5 cm depth) was measured continuously (every hour) in three drought and three control subplots from April 2016 to March 2017 using an EasyLog temperature logger (EL-USB-1, Lascar electronics, UK). Soil volumetric moisture (0–30 cm) was also measured continuously (every hour) in these subplots through time domain reflectometry (TDR) using a 30 cm long sensor and CR1000 data logger (Campbell Scientific Inc., USA).

2.3. Soil sampling

Soils (0–5 cm depth) were sampled in May 2017 from control and drought plots of 1, 2, 3 and 4 levels of TSA, with oak as the central species (Table 1). Samples were taken from three replicated plots, resulting in three independent replicates. In each plot, four soil cores were taken using a plastic corer (7 cm diameter), at points equidistant between the central oak tree and trees at each corner of the 3 × 3 tree plot (Supplementary Fig. S1). After removing the field layer, the top 5 cm of soils were retained for analysis. Soil from the four cores from each subplot were mixed together to form a composite sample. The soil was passed through a 4 mm sieve and plant and root material and other debris was removed.

Soil sub-samples were used to measure gravimetric soil water content (105 °C to constant mass) and soil organic matter (SOM) content through loss on ignition (600 °C for 12 h). Soil C and N content was determined using a C/N elemental analyser (Dumas combustion). Soil pH was measured in a 1:5 (w/v) soil:H_2O solution using a pH meter. Water holding capacity (WHC) was measured by weighing 5.0 g soil into a plastic tube, with the underside covered with fine nylon mesh (50 µm) to prevent loss of soil particles and the top covered with parafilm to prevent evaporation of water. The tubes were then placed in water for 24 h, before they were removed, allowed to drain for 6 h and re-weighed, to determine maximum WHC. The gravimetric water content of control and drought soil samples, when expressed as % WHC, were not statistically different (29.3 ± 1.3% and 26.6 ± 1.3% of WHC for control and drought, respectively; overall mean soil moisture 28 ± 1% WHC). Hence, soils were used without further adjustment to measure rates of bacterial growth, fungal growth, respiration and substrate induced respiration, as well as microbial phospholipid fatty acid (PLFA) composition.

2.4. Bacterial and fungal growth

Bacterial growth was determined by measuring the rate of ^{3}H-Leucine (Leu) incorporation in extracted bacteria (Bååth et al., 2001; Rousk et al., 2009). One gram fresh soil was mixed with 20 ml demineralized water, vortexed for 3 min and centrifuged (10 min at 1000 g). The resulting bacterial suspension was incubated at 15 °C for 1 h, with 2 µl 1-[4,5-^{3}H]-Leucine (5.7 TBq mmol^{-1}, Perkin Elmer, USA) and unlabelled Leu with a final concentration of 275 nM Leu in the bacterial suspension. Bacterial growth was terminated after 1 h by adding 75 µl of 100% trichloroacetic acid. Centrifugation and washing was performed as described by Bååth et al. (2001). Scintillation cocktail (Ultima Gold; PerkinElmer, USA) was added and the radioactivity was measured using a liquid scintillation counter. The amount of leucine incorporated into extracted bacteria (pmol Leu incorporated g^{-1} SOM h^{-1}) was used as a measure of bacterial growth.

Fungal growth was determined using the acetate-in-ergosterol (Ac-in-erg) incorporation method (Newell and Fallon, 1991) adapted for soil (Bååth et al., 2001; Rousk et al., 2009), which estimates the rate of ergosterol synthesis as a measure of fungal growth. One gram soil was mixed with 20 µl of ^{14}C-acetate solution ([1–^{14}C] acetic acid, sodium salt, 2.07 GBq mmol^{-1}, Perkin Elmer) and unlabelled sodium acetate, resulting in a final acetate concentration of 220 µM in the soil slurry. Samples were incubated at 15 °C for 2 h in the dark before growth was terminated by addition of formalin. Ergosterol and incorporated acetate were measured according to Rousk and Bååth (2007). The amount of acetate incorporated into ergosterol (pmol g^{-1} SOM h^{-1}) was used as a measure of fungal growth. Ergosterol concentration was estimated from the UV absorbance at 282 nm compared with external standards.

2.5. PLFA composition

Microbial PLFA composition was determined using 2.0 g frozen soil, according to Frostegård et al. (1993) with modifications (Nilsson et al., 2007). An internal standard (methyl nonadecanoate fatty acid 19:0) was added before the methylation step for quantification. The derived fatty acid methyl esters were quantified on a gas chromatograph with flame ionization detector. Bacterial (i15:0, a15:0, i16:0, 16.1ω9, 16.1ω7, i17:0, a17:0, cy17:0, 18:1ω7 and cy19:0) and fungal-specific (18:2ω6,9) PLFAs were used to estimate the relative abundance of these functional groups (Frostegård and Bååth, 1996; Ruess and Chamberlain, 2010). The sum total concentration of the PLFAs i14:0, 14:0, 15:0, 16:1ω5, 16:0, 17:1ω8, 17:0, 10Me17:0, 18:1ω9, 18:1, 18:0, 19:1 and 10Me18:0, in addition to those listed above as bacterial and fungal biomarkers, was used as a measure of total microbial abundance (Frostegård and Bååth, 1996; Ruess and Chamberlain, 2010).

2.6. Soil respiration and microbial biomass

One gram soil was weighed into 20 ml glass vials. The head space of glass vials was purged with pressurized air, before vials were sealed and incubated at 15 °C for 18 h. The amount of CO_2 produced during the incubation was determined using a gas chromatograph equipped with a methanizer and flame ionization detector. Substrate induced respiration was measured as a proxy for microbial biomass. Briefly, 30 mg 4:1 glucose:talcum was vigorously mixed into 2.0 g soil (corresponding to 4.8 mg glucose-C g^{-1} soil fwt). After 30 min, vials were purged with pressurized air and incubated at 15 °C for 2 h before the concentration of CO_2 was determined. Substrate induced respiration was used to estimate microbial biomass (mg C g^{-1} SOM) (Anderson and Domsch, 1978).

2.7. Data analysis

Main and interactive effects of drought treatment and TSA on soil physio-chemical properties, microbial process rates and microbial biomass parameters were tested by two-way analysis-of-variance (ANOVA). Prior to analysis, where necessary, dependent data were first log-transformed in order to meet the assumptions (homogeneity of variance) of ANOVA. Pairwise comparisons of significant effects were conducted using Tukey's HSD post hoc tests, with significant differences identified where $p < 0.05$.

To evaluate whether small differences in moisture among soils were related to the variation in microbial process rates and biomass, microbial parameters were regressed against both gravimetric soil water content and soil moisture as a percentage of WHC. In each case, regression analyses were conducted separately for control and drought-exposed soils to avoid the Simpson paradox (Gelman et al., 2007), using individual samples as independent data points ($n = 12$), with significant relationships identified where $p < 0.05$.

A principal component analysis (PCA) was used to screen for differences in the PLFA composition of the soil microbial community, using relative abundances (mol%) of PLFAs, after standardising to unit-variance. The scores of the principal components were subjected to two-way ANOVA (as above), and the variable loadings were used to interpret which PLFA markers explained separation of the principal components. Statistical analyses were performed using R, version 3.2.1 (R Core Team, 2015), and the PCA was performed using Multivariate Statistical Package (MVSP, Kovach Computing Services, Anglesey, Wales).

3. Results

3.1. Soil temperature and moisture

In the year prior to soil sampling, soil temperature (5 cm depth) ranged from 1 to 18 °C, with no difference between the control and drought plots evident from visual examination of the data (Fig. 1a). Rain exclusion shelters reduced incoming precipitation by 45–55%. To assess the relative reduction in soil moisture as a result of the rain exclusion shelters, the difference in moisture between drought and control soils was calculated as a percentage of soil volumetric water content in the control soils. Soil moisture (0–30 cm depth) was consistently lower in the drought plots compared to the control plots, whereby the rain exlusion shelters resulted in 14 ± 1 (mean ± SE) % lower mean volumetric soil water content in the drought plots relative to the control plots over the year prior to sampling (Fig. 1b). The most marked difference in soil moisture occurred between September-November 2016, where volumetric soil water content was 26 ± 2 (mean ± SE) % lower in the drought plots compared to the control. At the time of sampling in May 2017, however, volumetric soil water content was very similar in control and drought plots (Fig. 1b).

3.2. Soil physio-chemistry

Soil organic matter content, total C, total N and C/N ratio were unaffected by TSA and drought treatments (Table 2). There was also no significant effect of TSA and drought treatments on soil pH. At the time of our assessment, gravimetric soil water content was lower in soils with a history of reduced precipitation compared to controls ($F_{1,16} = 7.3$, $p = 0.02$), however there was no significant difference in soil moisture when expressed as a % of WHC between control and drought-exposed soils (Table 2). There was a significant effect of TSA on soil moisture as a % of WHC ($F_{3,16} = 4.6$, $p = 0.02$), with post-hoc pairwise comparisons showing that soil moisture was greater in soils with admixtures of four tree species compared to soils with admixtures of three tree species.

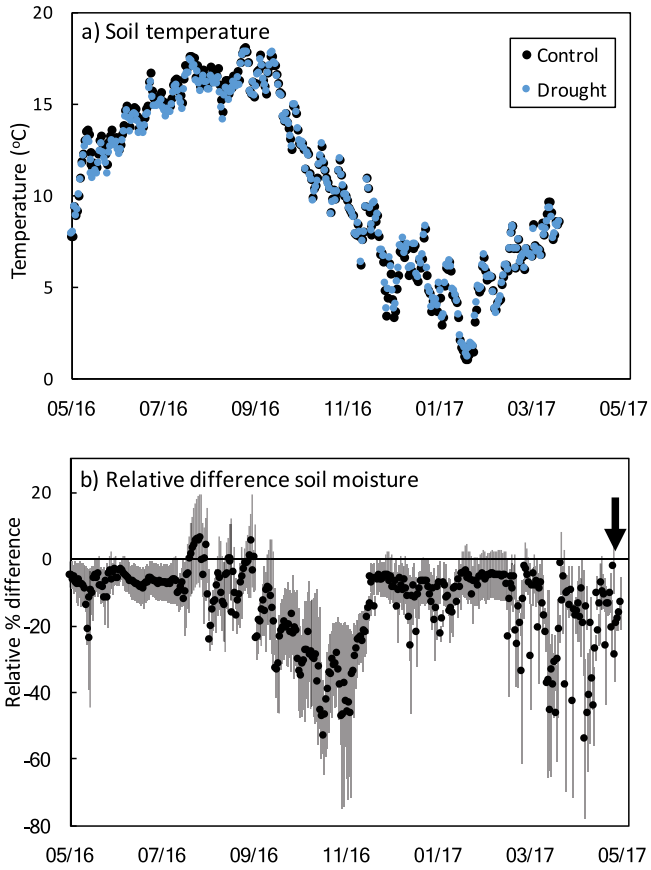

Fig. 1. (**a**) Soil temperature (5 cm depth) in control and drought plots and (**b**) the relative difference in soil moisture (volumetric soil water content; 0–30 cm depth) between control and drought plots during the year prior to soil sampling in May 2017. In panel b, values above zero indicate higher moisture in the drought plot relative to the control plot, and values below zero indicate lower moisture in the drought plot relative to the control plot. Data represent mean ± 1SE ($n = 3$), where error bars cannot be seen, the bar is smaller than the symbol. Arrow indicates time of soil sampling.

3.3. Tree species admixture and drought-legacy effect on microbial activity

Bacterial growth ($F_{1,16} = 7.6$, $p = 0.01$; Fig. 2a) and respiration ($F_{1,16} = 17.8$, $p < 0.001$; Fig. 2c) were significantly lower in drought-exposed soils, compared to control soils, while TSA had no significant effect. Fungal growth was not affected by a history of drought or differences in TSA (Fig. 2b). There was no significant interaction between TSA and drought treatment on bacterial growth, fungal growth or respiration.

There was a marginal interactive effect of drought and TSA on the fungal-to-bacterial growth ratio ($F_{3,16} = 2.8$, $p = 0.07$). When the number of different tree species in admixtures was low (monoculture or two tree species in admixture), the ratio of fungal-to-bacterial growth tended to be higher in soils with a history of drought, compared to the control (Fig. 2d). In contrast, in soils with greater tree admixing (three or four different tree species in admixture), there was no difference in the fungal-to-bacterial growth ratio between control soils and those with a history of drought. Differences in microbial process rates were not explained by small differences in soil moisture among soils, as the relationship between process rates and gravimetric soil water content ($p > 0.22$) or soil moisture (% WHC; $p > 0.19$; Supplementary Fig. S2) was not significant.

3.4. Microbial biomass

The total PLFA concentration ($F_{1,15} = 5.4$, $p = 0.04$), bacterial PLFA

Table 2
Soil physio-chemical characteristics in control and drought plots with different tree species admixtures (1–4 different tree species in admixture; see Table 1). Data represent mean (SE; $n = 3$). SWC = soil water content, WHC = water holding capacity.

	Control				Drought			
	1	2	3	4	1	2	3	4
Gravimetric SWC (g H_2O g^{-1} dwt)	0.19 (0.02)	0.18 (0.01)	0.20 (0.02)	0.21 (0.01)	0.16 (0.01)	0.15 (0.01)	0.16 (0.02)	0.20 (0.01)
Max. WHC (g H_2O g^{-1} dwt)	0.65 (0.05)	0.68 (0.07)	0.74 (0.04)	0.63 (0.03)	0.57 (0.04)	0.62 (0.03)	0.70 (0.06)	0.65 (0.03)
% WHC	30.0 (1.1)	26.6 (2.0)	26.9 (3.1)	33.6 (2.1)	28.6 (0.5)	24.5 (2.6)	22.7 (2.1)	30.4 (2.4)
Soil organic matter (%)	4.0 (0.09)	3.9 (0.09)	4.5 (0.42)	3.8 (0.04)	4.0 (0.25)	3.9 (0.08)	3.9 (0.29)	3.9 (0.15)
Soil C (%)	1.90 (0.16)	1.90 (0.14)	2.25 (0.20)	1.62 (0.09)	1.78 (0.19)	1.64 (0.02)	1.78 (0.15)	1.87 (0.07)
Soil N (%)	0.19 (0.01)	0.20 (0.01)	0.26 (0.04)	0.20 (0.01)	0.19 (0.02)	0.18 (0.01)	0.21 (0.02)	0.23 (0.02)
Soil C/N	9.9 (0.8)	9.4 (0.1)	8.9 (0.5)	8.0 (0.4)	9.2 (0.2)	9.2 (0.1)	8.4 (0.7)	8.3 (0.5)
Soil pH_{H2O}	6.4 (0.07)	6.2 (0.07)	6.3 (0.05)	6.3 (0.11)	6.4 (0.02)	6.1 (0.10)	6.4 (0.14)	6.3 (0.14)

Main and interactive effects of drought treatment (D) and tree species admixture (A) on: **Gravimetric SWC** (D) $F_{1,16} = 7.3$, $p = 0.02$; (A) $F_{3,16} = 2.4$, $p = 0.1$; (D x A) $F_{3,16} = 0.2$, $p = 0.9$; **Max. WHC** (D) $F_{1,16} = 1.4$, $p = 0.3$; (A) $F_{3,16} = 2.0$, $p = 0.2$; (D x A) $F_{3,16} = 0.4$, $p = 0.8$; **% WHC** (D) $F_{1,16} = 3.0$, $p = 0.1$; (A) $F_{3,16} = 4.6$, $p = 0.02$; (D x A) $F_{3,16} = 0.2$, $p = 0.9$; **Soil organic matter** (D) $F_{1,16} = 0.5$, $p = 0.5$; (A) $F_{3,16} = 0.6$, $p = 0.6$; (D x A) $F_{3,16} = 1.1$, $p = 0.4$; **Soil C** (D) $F_{1,16} = 2.1$, $p = 0.2$; (A) $F_{3,16} = 1.3$, $p = 0.3$; (D x A) $F_{3,16} = 2.3$, $p = 0.1$; **Soil N** (D) $F_{1,16} = 0.8$, $p = 0.4$; (A) $F_{3,16} = 2.5$, $p = 0.09$; (D x A) $F_{3,16} = 1.3$, $p = 0.3$; **Soil C/N** (D) $F_{1,16} = 0.8$, $p = 0.4$; (A) $F_{3,16} = 2.5$, $p = 0.09$; (D x A) $F_{3,16} = 1.3$, $p = 0.3$; **Soil pH** (D) $F_{1,15} = 0.3$, $p = 0.6$; (A) $F_{3,15} = 2.8$, $p = 0.08$; (D x A) $F_{3,15} = 0.3$, $p = 0.8$.

concentration ($F_{1,15} = 5.5$, $p = 0.03$) and fungal PLFA concentration ($F_{1,15} = 5.4$, $p = 0.04$) were significantly lower in soils with a history of drought (Table 3). TSA did not affect the total concentration of PLFAs or bacterial PLFAs, but did affect the concentration of fungal PLFAs ($F_{3,15} = 3.6$, $p = 0.04$). In this case, post-hoc pairwise comparisons showed that soils with admixtures of four different tree species had a lower concentration of fungal PLFAs compared to soils with admixtures of three different tree species. Microbial biomass C, determined by substrate induced respiration ($F_{1,16} = 5.9$, $p = 0.03$), and fungal biomass, determined from ergosterol concentration ($F_{1,16} = 14.6$, $p = 0.002$) were also lower in soils with a history of drought, but were unaffected by TSA. There were no positive relationships between microbial biomass parameters and soil gravimetric water content ($p > 0.09$) or soil moisture (% WHC; $p > 0.18$; Supplementary Fig. S3).

3.5. Microbial community

The first principal component (PC1) from a PCA of microbial PLFA composition explained 31.2% of the variation in the data, while the second principal component (PC2) explained another 19.9% (Fig. 3). Microbial community composition was strongly affected by TSA ($F_{3,15} = 8.3$, $p = 0.002$ for PC1). Post-hoc pairwise comparisons showed that PC1 scores for soils under oak monoculture and admixtures of two different tree species were significantly different to PC1 scores for soils with admixtures of three and four different tree species (Fig. 3a). Differences along PC1 appeared to be related to higher relative abundances of PLFA markers associated with gram-negative bacteria (including cy17:0, 18:1ω7 and cy19:0) towards negative variable loadings and higher relative abundance of the fungal marker 18:2ω6,9 towards positive variable loadings (Fig. 3b). In the case of PLFA markers associated with gram-positive bacteria, there was a greater relative abundance of i15:0 and a15:0 markers towards positive variable loadings and a greater relative abundance of i16:0, i17:0 and a17:0 markers towards negative variable loadings. There was no significant effect by drought treatment on microbial community structure, and no interactive effect of TSA and drought.

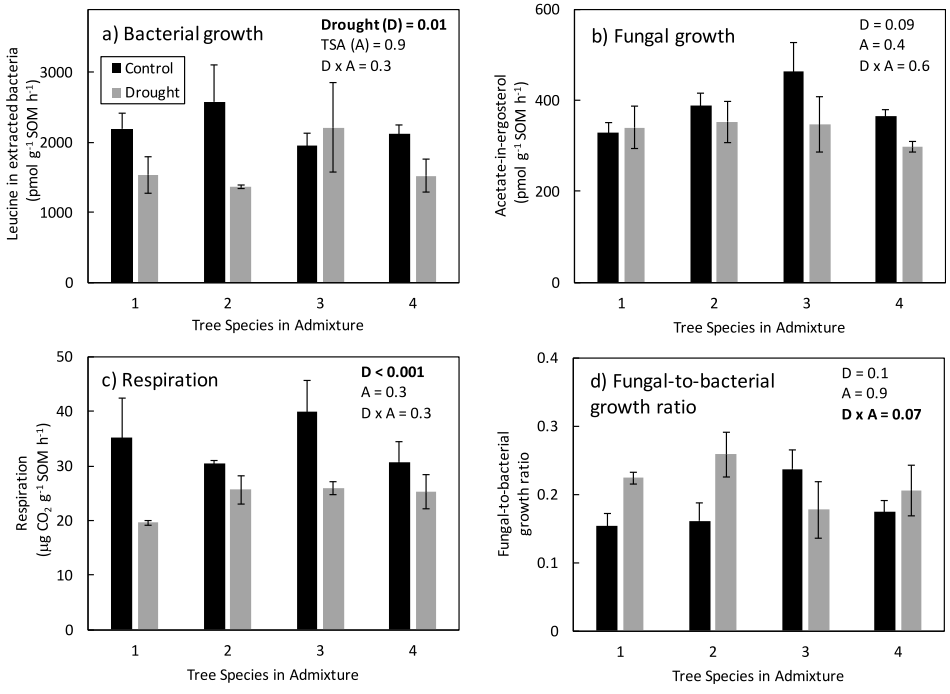

Fig. 2. The effect of drought (D) and tree species admixture (A) on (**a**) bacterial growth, (**b**) fungal growth, (**c**) respiration and (**d**) fungal:bacterial growth ratio. Bars represent mean ± 1SE ($n = 3$). Results of two-way ANOVA (p values) presented, with significant and marginally significant results highlighted in bold. See Table 1 for tree species composition in admixture.

Table 3
Microbial biomass parameters in control and drought plots with different tree species admixtures (reported per g soil organic matter; SOM). Data represent mean (SE; $n = 3$).

	Control				Drought			
	1	2	3	4	1	2	3	4
Microbial biomass (mg C g^{-1} SOM)	6.9 (0.6)	6.8 (0.6)	7.2 (0.3)	6.5 (0.1)	5.5 (0.7)	6.5 (0.6)	6.4 (0.3)	5.6 (0.4)
Total PLFA (nmol g^{-1} SOM)	1697 (248)	1528 (163)	1520 (56)	1324 (135)	1119 (124)	1395 (33)	1299 (128)	1314 (71)
Fungal PLFA (nmol g^{-1} SOM)	72.3 (12.3)	63.8 (4.9)	75.2 (8.7)	54.4 (13.0)	55.3 (10.7)	58.9 (3.7)	57.1 (4.3)	38.6 (5.7)
Bacterial PLFA (nmol g^{-1} SOM)	769 (125)	706 (80)	702 (38)	620 (83)	501 (57)	654 (19)	587 (61)	573 (19)
Ergosterol (μg g^{-1} SOM)	128 (22)	139 (31)	144 (10)	114 (5)	95 (18)	113 (14)	87 (13)	61 (7)

Main and interactive effects of drought treatment (D) and tree species admixture (A) on: **Microbial biomass** (D) $F_{1,16} = 5.9$, $p = 0.03$; (A) $F_{3,16} = 1.0$, $p = 0.4$; (D x A) $F_{3,16} = 0.5$, $p = 0.7$; **Total PLFA** (D) $F_{1,15} = 5.4$, $p = 0.04$; (A) $F_{3,15} = 0.5$, $p = 0.7$; (D x A) $F_{3,15} = 1.7$, $p = 0.2$; **Fungal PLFA** (D) $F_{1,15} = 5.4$, $p = 0.04$; (A) $F_{3,15} = 3.6$, $p = 0.04$; (D x A) $F_{3,15} = 0.4$, $p = 0.8$; **Bacterial PLFA** (D) $F_{1,15} = 5.5$, $p = 0.03$; (A) $F_{3,15} = 0.6$, $p = 0.6$; (D x A) $F_{3,15} = 1.2$, $p = 0.3$; **Ergosterol** (D) $F_{1,16} = 14.6$, $p = 0.002$; (A) $F_{3,16} = 2.3$, $p = 0.1$; (D x A) $F_{3,16} = 0.9$, $p = 0.4$.

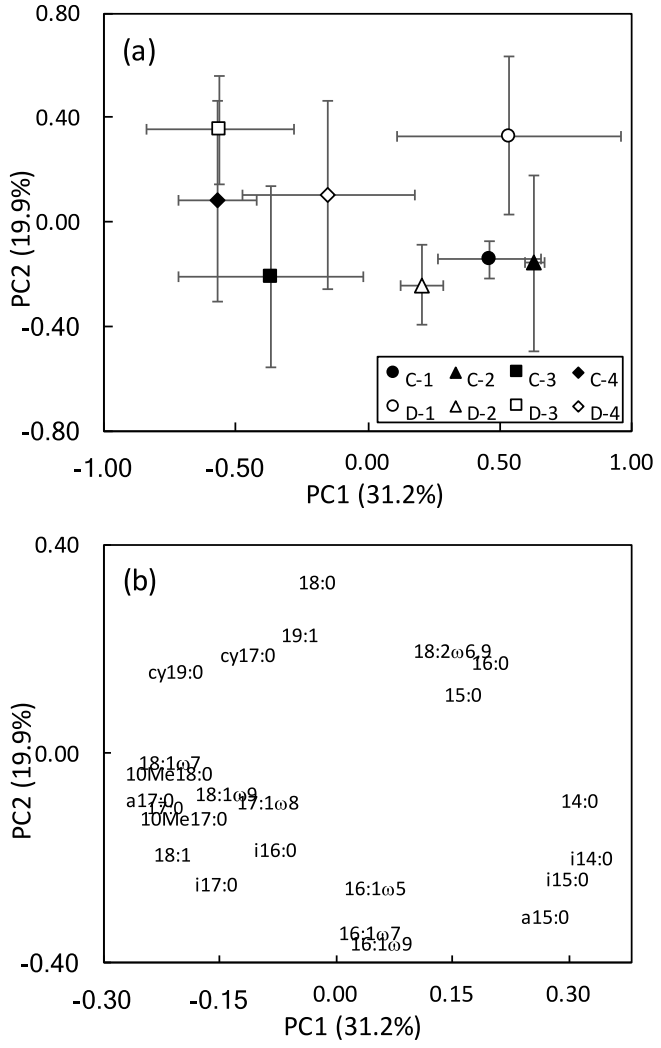

Fig. 3. Microbial community composition in soils dependent on tree species admixture (1–4 different tree species in admixture) and drought treatment (C = control, D = drought), according to a principal component analysis of the PLFA composition (expressed as mol%). (**a**) scores of the first two principal components, together explaining 51.1% variation (data represent mean ± 1SE, $n = 3$). (**b**) loadings of the first two components from the PCA of PLFA composition, showing the PLFA markers driving the separation among the samples in panel a.

4. Discussion

4.1. Field experiment effects

Drought events are expected to intensify over the 21st Century (IPCC, 2013), with the potential to influence both plant and microbial communities. We investigated the legacy effect of drought on microbial activity, biomass and composition, and tested whether mixed planting mitigated against the effect of drought on soil microorganisms, by using soils from an experimental plantation in Belgium, under reduced precipitation. As such, our assessment relied on the efficacy of the field drought treatment and controlled tree species composition in the experimental plantation to have an impact on the belowground microbial community. Mixed planting in the experimental plantation lead to significant differences in microbial community composition (Fig. 3), likely driven by differences in the physio-chemical traits of litter and root inputs from different species of plant (Wardle et al., 2004; Thoms et al., 2010; Carnol and Bazgir, 2013). In particular, the presence of pine trees in some of the three- and four-species admixture plots may explain this response, as leaf-litter from coniferous trees is typically more recalcitrant compared to litter from broadleaf trees (Berg and McClaugherty, 2013; Setiawan et al., 2016). Rain shelters, excluding c. 50% of incoming precipitation, were also effective, reducing volumetric soil water content by an average of 14% over the year prior to soil sampling (Fig. 1b). At the time of our microbial assessment, however, soil moisture was not different in control and drought-exposed soils (Table 2). Moreover, there was no positive relationship between any measured microbial parameter and soil moisture, indicating no direct effect of moisture content on microbial activity or biomass during our assessment. Thus, the criteria for evaluating our hypotheses were met, enabling us to evaluate the legacy effect of drought and mixed planting on current process rates.

4.2. Drought-legacy effects on current microbial process rates

As we hypothesised, bacterial growth and respiration were lower in soils with a legacy of drought (Fig. 2a and 2c). This response could not be attributed to a change in any physio-chemical parameter that we measured, as important determinants of microbial process rates such as pH and SOM concentrations were unaffected by the drought treatment (Table 2). Other studies have also reported drought-legacy effects on soil respiration (Evans and Wallenstein, 2012; Göransson et al., 2013; Hawkes et al., 2017). In one forest study, a history of drought resulted in lower rates of respiration following rewetting, with this response attributed to lower labile C availability in historically drought-exposed soils, as a consequence of reduced C input from plants during drought (Göransson et al., 2013). A similar mechanism could explain our findings. Respiration per unit SOM is used an index of C quality, reflecting both the availability and lability of C substrate mineralised by soil

microorganisms at a given time, assuming no difference in microbial C allocation between growth and respiration (Fierer et al., 2006; Conant et al., 2011). Lower respiration per unit SOM therefore suggests that the 'quality' of C in drought-exposed soils was lower than in control soils (Fig. 2c). As such, reduced plant input during drought, resulting in a lower availability of labile C, could explain the lower bacterial growth in drought-exposed soils. Alternatively, in another study, a drought-legacy effect on litter decomposition was explained by lower microbial abundance and differences in microbial composition driven by drought (Allison et al., 2013). In our study, however, there was no clear difference in microbial community composition between control and drought-exposed soils (Fig. 3). In contrast to our findings, a previous study of pan-European soils found that history of drought did not affect rates of microbial growth or respiration (Rousk et al., 2013). In this case, however, the study focused on shrubland rather than forest plantation, and the drought treatment was milder, with only 30% precipitation reduction during the summer growing season compared to 50% precipitation reduction throughout the year in our study. This contrast could suggest a non-linear response where microbial responses to drought are subject to a threshold level (Lenton, 2011).

4.3. Differential drought-legacy effects on bacteria and fungi

In support of our hypothesis that bacteria are more sensitive to drought, we observed lower bacterial growth in soils with a history of drought (Fig. 2a), while fungal growth was unaffected (Fig. 2b). Bacteria are often considered to be more dependent on the labile C input from plants (Singh et al., 2006; Andresen et al., 2014), which can be reduced during drought (Ciais et al., 2005; Ruehr et al., 2009). Consistent with this expectation, experimental drought reduced the C allocation from plants to bacteria, but not fungi, in an Austrian mountain meadow (Fuchslueger et al., 2014). Long-term drought in Mediterranean forest and shrubland systems affected the bacterial rather than fungal community, with decreased bacterial but not fungal diversity (Yuste et al., 2011). Bacterial growth was also severely reduced compared to fungal growth during repeated drying-rewetting cycles in a grassland soil (Bapiri et al., 2010). Moreover, in a study of agricultural soils, fungal-dominated soils were found to be more resistant to drought compared to bacterial-dominated soils (de Vries et al., 2012). Together, these findings suggest that bacterial communities will be most vulnerable to future drought, in part due to their reliance on plant derived labile C which is reduced during drought.

Microbial process rates measured here provided a snapshot of microbial function under stable conditions, enabling us to evaluate how a legacy of drought (i.e. the indirect rather than direct effects of drought) influenced current process rates (Fig. 2). In contrast, microbial biomass parameters integrated the recent history of environmental conditions, and as such, reflect both the direct (reduction in soil moisture) and indirect (reduced C input) effects of drought. In support of our hypothesis, microbial biomass was consistently lower in drought-exposed soils (Table 3), indicating that the recent history of lower water availability, and potentially reduced plant input, under experimental drought had resulted in a smaller biomass pool. This finding is consistent with recent meta-analyses that also found that large precipitation reductions decreased total soil microbial biomass (Canarini et al., 2017; Homyak et al., 2017; Ren et al., 2018). Interestingly, despite lower biomass in drought-exposed soils, microbial community composition was unaffected by the drought treatment (Fig. 3), suggesting that the whole microbial community was similarly affected by drought, with reduced total abundance but no change in the relative abundance of different microbial PLFAs. Indeed, while a legacy of drought did not affect fungal growth rates under stable conditions in our study, both fungal and bacterial biomass were lower in soils with a history of drought (Table 3). As there was no difference in fungal growth between control and drought-exposed soils when measured at the same soil moisture content (Fig. 2b), there is no evidence that fungi were affected by indirect effects of drought, such as reduced plant input. The direct effect of low moisture availability is, therefore, likely to have been responsible for a smaller build-up of fungal biomass under drought. Fungi are often considered to be more tolerant to moisture stress, because their chitinous cell wall increases protection against low water availability (Harris, 1981; Manzoni et al., 2012) while their filamentous structure allows them to translocate and redistribute water in soils (Guhr et al., 2015). However, our results suggest that both fungi and bacteria were similarly susceptible to the direct effect of reduced water availability under drought, resulting in reduced biomass of both groups.

4.4. Mixed planting may mitigate drought-legacy effect

Some studies have observed that forests with greater species mixing are more resistant to drought (Lebourgeois et al., 2013; Pretzsch et al., 2013; Mina et al., 2017). Greater plant productivity, and consequently higher plant input to soil, during drought in mixed stands compared to monoculture may, therefore, lead to less pronounced drought-legacy effects. While mindful of the risk of false positives which may arise from multiple statistical tests, here we observed a marginal interactive effect of drought and TSA on the ratio of fungal-to-bacterial growth in soils (Fig. 2d). This response appeared to be driven by the decrease in bacterial growth in soils of lower admixing (monoculture and two-species admixtures) with a legacy of drought, which resulted in a higher ratio of fungal-to-bacterial growth in these soils. This result supports our hypothesis, suggesting that bacterial growth was disproportionally influenced by mixed planting in the admixtures included in our study (Table 1). One explanation may be that drought more strongly weakened the flow of plant input to soils in oak monoculture and two-species admixture plots, resulting in reduced availability of labile C and consequently lower bacterial growth in these soils. Few studies have assessed whether mixed planting can mitigate the effects of drought on soil microorganisms. In one grassland study, there was no interactive effect of plant diversity and drought on litter mass loss (Vogel et al., 2013). In another grassland study, the response of enzyme activity under drought depended on plant composition, whereby enzyme activity was typically lower in mixed planted soils compared to monoculture (Sanaullah et al., 2011). Our findings, however, suggest that admixing oak with other tree species may help to mitigate against drought effects on soil microorganisms. We can also add some quantitative precision to the observed effects, noting that admixing oak with 2–3 species could provide a 25% amelioration of the legacy effect of 50% precipitation reduction in the studied ecosystem. Naturally, this observation will require further verification. In particular, whether this response was driven by the presence of a particular tree species (with specific functional traits) in the admixture, or increased tree richness in general, merits investigation, as complementarity effects of mixed planting under drought can be strongly influenced by species composition (Mina et al., 2017).

5. Conclusion

Our results show that drought can have lasting legacy effects on soil microorganisms, with consequences for microbial functions. Bacteria were more adversely affected by a history of drought compared to fungi, which may reflect the fact that bacteria are more dependent on labile C input from plants, which is often reduced during drought. Although bacteria were more sensitive to the indirect effect of drought compared to fungi, we found that both fungal and bacterial biomass was lower in drought-exposed soils, suggesting that the direct effect of reduced moisture affected both groups similarly. Results also suggest that admixing oak with other tree species, to increase tree functional traits and thus niche complementarity, may be a strategy to alleviate the drought-legacy effect on bacteria and build up tolerance to future drought.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2018.05.027.

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